Adenosine Diphosphate as a Regulatory Ligand in Beef Heart Cytosol Nucleoside Diphosphokinase†

Maurice G. Colomb,* Arlette Chéruy, and Pierre V. Vignais

With the Technical Assistance of A. M. Duplaa and C. Meyer

ABSTRACT: Nucleoside diphosphokinase (NDP-kinase) from beef heart cytosol has been crystallized. The enzyme is an oligomeric protein made of six subunits with a similar molecular weight (16,000–18,000). One mole of native NDP-kinase binds up to 6 mol of *p*-chloromercuribenzoate, suggesting that each subunit possesses only one free sulfhydryl group. However, NDP-kinase incubated with [^{32}P]ATP does not bind more than 3 mol of phosphate/mol of enzyme. Dissociation of the hexamer into subunits is a reversible process. Treatment with sodium dodecyl sulfate (SDS), citraconic anhydride, or maleic anhydride allows the isolation of subunits which will reassociate to regenerate enzymatically active hexamer after elimination of the dissociating reagent. When dissociation by citraconic anhydride is carried out in the presence of ATP, more dimers than monomers are formed, suggesting that the dimers may reflect a functional association of monomers. The transphosphorylation between nucleoside tri- and diphosphates catalyzed by native NDP-kinase is strongly and selectively inhibited by ADP; the K_i for ADP is three to five times less than the K_M for ADP. Treatment by SDS at concentrations which do not dissociate the enzyme, or by small amounts of *p*-chloromercuribenzoate, specifically increases the K_i^{ADP}. The increasing effect of SDS on K_i^{ADP} is accompanied by the appearance of a pH dependence of the photoinactivation of NDP-kinase, and by a significant increase of the fluorescence polarization of the tryptophan residues. A modified Ping-Pong mechanism, which includes an ADP regulatory site or two interacting catalytic sites, is proposed to explain the selective inhibitory effect of ADP.

Nucleoside diphosphokinase is a wide-spread enzyme, that has been purified from yeast, *Saccharomyces carlbergensis* (Ratliff *et al.*, 1964) and *Saccharomyces cerevisiae* (Edlund *et al.*, 1969), and from liver (Pedersen, 1968), heart (Colomb *et al.*, 1972), erythrocytes (Mourad and Parks, 1966), pea seed (Edlund, 1971), and bacteria, *Bacillus subtilis* (Sedmak and Ramaley, 1971) and *Rhodospirillum rubrum* (Yamamoto *et al.*, 1972). In spite of slight differences in physical properties and in specificity, all the NDP-kinases[1] so far isolated display the same Ping-Pong mechanism characterized by a two-step reaction with two stable forms of the enzyme. In the first step, a nucleoside triphosphate, NTP, transfers its terminal phosphate group to the free enzyme E to yield phosphorylated enzyme EP with release of NDP; EP then discharges its phosphate onto another nucleoside diphosphate N′DP with regeneration of E. These partial reactions are summarized in eq 1 and 2.

$$NTP + E \rightleftarrows EP + NDP \quad (1)$$

$$N'DP + EP \rightleftarrows E + N'TP \quad (2)$$

In the cell, the main function of NDP-kinase is to catalyze the transfer of phosphate from ATP to nucleoside diphosphates other than ADP and to replenish the cellular cytosol with UTP, CTP, and GTP.

The Ping-Pong mechanism predicts that competitive substrate inhibition should result from combination of reactants with the wrong stable enzyme form to give abortive complexes (Cleland, 1963). However, the ATP–NDP transphosphorylation is inhibited to a different extent according to the NDP species used. For example, in the case of NDP-kinase from rat liver mitochondria, ADP and GDP are more inhibitory than other nucleoside diphosphates (Goffeau *et al.*, 1968; Pedersen, 1973). As shown in a previous paper, ADP inhibits the ADP–ATP exchange catalyzed by beef heart NDP-kinase by competing with ATP for the free enzyme (Colomb *et al.*, 1972a). Recent investigations in this laboratory carried out with a crystallized preparation of beef heart cytosol NDP-kinase have revealed: (1) that ADP is a more effective inhibitor than other nucleoside diphosphates tested, (2) that the K_i value for ADP is three to five times less than the K_M for ADP, and (3) that chemical treatment of the enzyme results in a differential increase of K_i^{ADP}, K_M^{ADP}, and K_M^{ATP} in the ATP–ADP exchange; in particular, K_i^{ADP} increases several fold before any variation of K_M^{ADP} or K_M^{ATP} is noticed. The latter effect recalls the desensitization mechanism in allosteric enzymes and suggests that NDP-kinase might contain two binding sites for ADP, one being the catalytic site involved in the Ping-Pong reaction, the other being a regulatory site. To evaluate the meaning of the selective increase of K_i^{ADP}, a study of the quaternary structure of beef heart NDP-kinase has been carried out in an attempt to detect conformational changes paralleling the kinetic modifications. A preliminary report of this work has been presented (Colomb *et al.*, 1973).

† From Laboratoire de Biochimie, Département de Recherche Fondamentale du Centre d'Etudes Nucléaires et Université Scientifique et Médicale de Grenoble, and from Laboratoire d'Automatique, Institut National Polytechnique de Grenoble, Grenoble, France. *Received October 29, 1973.* This investigation was supported by research grants from the Centre National de la Recherche Scientifique, the Fondation pour la Recherche Médicale and the Délégation Générale à la Recherche Scientifique et Technique.

[1] Abbreviations used are: NDP and NTP, nucleoside di- and triphosphate; SDS, sodium dodecyl sulfate; NDP-kinase, nucleoside diphosphokinase or nucleoside triphosphate: nucleoside diphosphate transphosphorylase, EC 2.7.4.6; phospho-NDP-kinase, phosphorylated derivative of NDP kinase; BSA, bovine serum albumin; pCMB, *p*-chloromercuribenzoate.

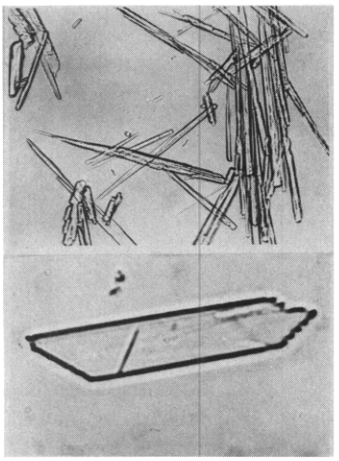

FIGURE 1: Crystals of beef heart cytosol NDP-kinase. Length of the crystals between 0.1 and 0.4 mm.

Experimental Section

Materials. The different nucleotides used were checked for contaminants. When necessary, they were purified either by chromatography according to Hurlbert (1957) or for very small quantities, by chromatography according to Duée (1968). [β-^{32}P]ADP and [γ-^{32}P]ATP were prepared as described in a previous paper (Colomb *et al.*, 1972a). Phenylmethylsulfonyl fluoride (B grade) was purchased from Calbiochem (Los Angeles, Calif.), maleic anhydride was obtained from Merck, and citraconic anhydride from Schuchardt (München, Germany). All other reagents were of the best grade commercially available.

Enzyme Assays. An isotopic test (Colomb *et al.*, 1969) was used for kinetic studies. In routine assays, the incubation medium consisted of 6 mM ATP, 12 mM $MgCl_2$, 0.2 mM [^{32}P]ADP, and 0.1 M triethanolamine-HCl buffer (pH 8.0) (standard incubation medium). Incubations were carried out for 5 or 10 min at 28°. For specific tests, the concentrations of ADP and ATP, or other nucleotides, were varied while the concentration of free Mg^{2+} was kept constant (Colomb *et al.*, 1972a). v is defined as the reaction velocity in the assay conditions, without correction; units of enzyme activity represent the number of μmoles of phosphate transferred per minute from nucleotide triphosphate to nucleotide diphosphate. V_{max} (velocity corrected for the inhibitory effect of ADP) was obtained from a secondary plot as described in Results.

A spectrophotometric assay was used for rapid scanning of enzyme activity at the various steps of the purification (Colomb *et al.*, 1972a).

Crystallization. The first steps of purification including the carboxymethyl (CM)-cellulose chromatography were carried out as previously described (Colomb *et al.*, 1972a). The specific activity of the CM-cellulose eluate was between 200 and 300 units/mg of protein. Further purification was achieved as follows. The CM-cellulose eluate was treated with ammonium sulfate according to Jakoby (1971). Saturated ammonium sulfate was added to the eluate to make it 66% of saturation at 0°. After 30 min, the resulting precipitate was collected by centrifugation for 10 min at 20,000g. The pellet was white and contained about 90% of the total NDP-kinase activity. The following steps were performed according to the procedure described by Ratliff *et al.* (1964) for yeast NDP-kinase, with minor modifications. The pellet was washed three times with 70% saturated ammonium sulfate and 10 mM EDTA (pH 6.5) and finally dissolved in 1 ml of 10 mM EDTA (pH 6.5). This solution which showed a specific activity ranging from 1000 to 2500 units/mg of protein according to preparations was dialyzed for 24 hr against 1 l. of 5 mM ammonium succinate (pH 6.5) with three changes of dialysis solution. Crystallization was initiated by addition of 70% ethanol to a final concentration of 25%. After standing at −10° for periods ranging from 12 hr to several days, fine needles appeared which were collected by gentle centrifugation at 3000g (Figure 1). No attempt was made to grow larger crystals. They were dissolved in the working buffers and used for the various experiments reported in this paper; they showed the same specific activity as that of the solution before crystallization.

Acrylamide Gel Electrophoresis. Electrophoresis in the absence of SDS was performed essentially as described by Shuster (1971) using 25 mM Tris–glycine (pH 8.3) as gel and reservoir buffer (unless indicated); 7.5% gels were prepared with an acrylamide to bisacrylamide ratio of 29 in plastic tubes of 11 × 0.6 cm. A prerun of 30 min was performed to get rid of ammonium persulfate. The samples (30 to 50 μl) in 10% sucrose containing Bromophenol Blue as indicator dye were loaded by layering on top of the gel in the tubes. The usual conditions of electrophoresis were 4 mA per tube with 250 V. The proteins were stained with Coomassie Blue as described by Gabriel (1971). Radioactivity measurements of gels are described in the following section.

SDS acrylamide gel electrophoresis was carried out according to Weber *et al.* (1972) for the estimation of molecular weights; 25 mM Tris–glycine buffer (pH 8.3) (Shuster, 1971) and 0.1% SDS were used as reservoir and gel buffer. The protein samples were treated with 1% SDS and 1% mercaptoethanol in sealed tubes for 1 hr at 28°. Bromophenol Blue (0.01%) was added together with sucrose (10%) before layering the samples on the gel tubes (0.6 cm × 11 cm). Marker proteins were treated in parallel. The following reference values were used: horse heart cytochrome c 11,700, BSA mol wt 68,000 (Weber *et al.*, 1972); rabbit muscle lactate dehydrogenase subunits mol wt 35,000, creatine-kinase subunits mol wt 40,000, horse hemoglobin subunits mol wt 16,000 (Darnall and Klotz, 1972). The electrophoresis was run with 250 V and 4 mA per tube and stopped when Bromophenol Blue reached the extremity of the tube. Because of some deviation from linearity for the migration of low molecular weight components (mol wt <20,000), particular care was taken to evaluate the comparative migration of reference proteins in order to ascertain the molecular weight of NDP-kinase subunits. Scanning of the gels was performed at 540 nm. With radioactive samples, the gels were frozen in Dry Ice powder and cut into 2-mm slices. ^{32}P radioactivity was counted on planchets, and ^{14}C activity in a scintillation medium as described in next section.

Binding of [^{14}C]pCMB. Gel electrophoresis was used to separate free from bound [^{14}C]pCMB after reaction of [^{14}C]-pCMB with NDP-kinase. Since the binding of pCMB to SH groups is a reversible process, the accurate measurement of –SH groups in NDP-kinase by [^{14}C]pCMB binding requires adequate corrections. We calculated appropriate corrections, based on the finding that the release of bound pCMB could be approximated as a first-order process. The acrylamide gel electrophoresis technique was adapted to the determination of the total amount of reactive –SH groups. The reaction mixture containing the enzyme and [^{14}C]pCMB was applied to a number of gels in the same run and electrophoresed to separate the free and bound [^{14}C]pCMB. Tubes were removed at various times from 30 to 90 min. The gels were sliced, then

incubated overnight at 60° in 1 ml of 5% SDS and 0.5 M sodium hydroxide in capped vials; 10 ml of scintillation fluid (100 g of naphthalene, 6 g of 2,5-diphenyloxazole, and 300 mg of *p*-bis(2-(5-phenyloxazolyl))benzene per liter of 1,4-dioxane) was added and the radioactivity determined. Radioactivity corresponding to the protein peak was summed. Extrapolation to zero electrophoresis time according to a semilogarithmic plot yielded the initial value of bound [^{14}C]-pCMB.

Binding of [^{32}*P*]*Phosphate.* [^{32}P]Phospho-NDP-kinase was prepared by reaction of NDP-kinase with [^{32}P]ATP and separated from unreacted [^{32}P]ATP by gel electrophoresis. For accurate measurement of the amount of bound [^{32}P]-phosphate, a correction was required for the time-dependent decay of bound radioactivity, due to the lability of the phosphate bond in phospho-NDP-kinase. Advantage was taken of the fact that this decay is first order. The reaction mixture containing [^{32}P]phospho-NDP-kinase and [^{32}P]ATP was submitted to gel electrophoresis in different tubes for different periods of time from 30 min to 2 hr. The gels were sliced and the ^{32}P radioactivity corresponding to [^{32}P]phospho-NDP-kinase was determined. Extrapolation to zero electrophoresis time according to a semilogarithmic plot yielded the corrected value of bound [^{32}P]phosphate.

Sucrose gradient centrifugation was performed as described by Martin and Ames (1961), with minor modifications (see Colomb *et al.*, 1969).

Maleylation and citraconylation of NDP-kinase was as described by Butler *et al.* (1969) and Dixon and Perham (1968).

Photooxidation was performed as described by Westhead (1972) at 25° with 0.01 mM Rose Bengal and 0.1 mM EDTA.

Fluorescence Measurements. Fluorescence intensity and fluorescence polarization were measured with a Perkin-Elmer MPF-2A spectrofluorimeter. All measurements were carried out at 15°. For intrinsic fluorescence, the excitation wavelength of 292 nm corresponded to the peak in the excitation spectrum. The emission wavelength (350 nm) was beyond the maximum which appears at 330–340 nm in order to avoid interference from the fluorescent analogs of adenine nucleotides (etheno-ADP and etheno-ATP used in other experiments not reported here). In these conditions, the fluorescence recorded was mainly due to tryptophan residues. The degree of fluorescence polarization of NDP-kinase (p) was calculated from the formula: $(I_{vv} - GI_{vh})/(I_{vv} + GI_{vh})$ where G is a correction factor applied to compensate for dispersion of the emitted light ($G = I_{hv}/I_{hh}$) and v and h refer to vertical and horizontal orientations of the polarizer and analyzer (Chen *et al.*, 1969).

Results

Subunit Organization. Beef heart cytosol NDP-kinase is characterized by a sedimentation coefficient of 5.9 S and by a molecular weight of 103,000–108,000 (Colomb *et al.*, 1972a). It is dissociated into subunits by a number of reagents, including SDS, maleic anhydride, and citraconic anhydride.

The molecular weight of NDP-kinase subunits estimated by SDS polyacrylamide gel electrophoresis after preincubation in 1% SDS and 1% mercaptoethanol is 16,000–18,000 (Figure 2). This value was not influenced by the addition of phenylmethylsulfonyl fluoride, a proteolytic inhibitor (*cf.* Weber *et al.*, 1972). A similar molecular weight for phosphorylated subunits was found when the SDS treatment was applied to [^{32}P]phospho-NDP-kinase. These data indicate

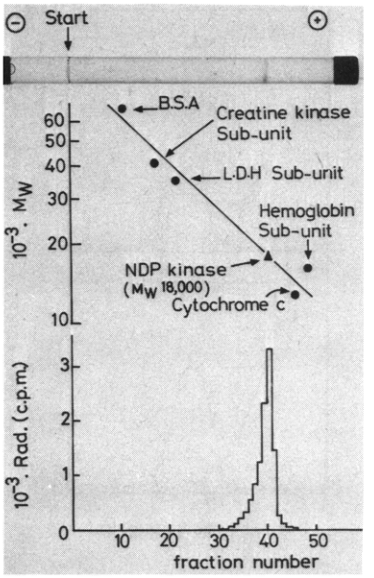

FIGURE 2: SDS acrylamide electrophoresis of beef heart NDP-kinase. From top to bottom: (a) Electrophoresis of NDP-kinase subunits: 5 μg of NDP-kinase in 50 μl of 30 mM phosphate buffer (pH 8) was layered on a 10% acrylamide gel after incubation in 1% SDS and 1% mercaptoethanol for 60 min at 28°. For other conditions see Methods. (b) Electrophoresis of reference protein subunits (same conditions as in (a)). (c) Electrophoresis of [^{32}P]-phospho-NDP-kinase subunits: 5.7 μg of NDP-kinase was incubated for 5 min at 28° with 2 μmol of [^{32}P]ATP (640,000 cpm/nmol) in 30 mM phosphate buffer (pH 8.0). SDS and mercaptoethanol were then added to a concentration of 1%. After an incubation of 60 min at 28° the medium was layered on the gel as in (a) and ^{32}P radioactivity was measured as described in the Experimental Section.

that beef heart cytosol NDP-kinase is an oligomeric protein with six subunits of identical molecular weight, like yeast NDP-kinase (Palmieri *et al.*, 1973).

Sulfhydryl groups in NDP-kinase were determined in the absence of denaturant with [^{14}C]pCMB as described in Methods. NDP-kinase (13 μg in 0.25 ml) was incubated for 1 hr at 20° with 1.5×10^{-4} pCMB in 15 mM Tris buffer (pH 8). Two different experiments yielded values of 4.8 and 6.2 nmol of [^{14}C]pCMB bound per nanomole of enzyme suggesting that 1 mol of NDP-kinase binds 6 mol of [^{14}C]pCMB and that one SH residue per monomer is readily titrated in native NDP-kinase. The binding of pCMB was totally reversed by treatment with 1 mM mercaptoethanol. In contrast with other oligomeric enzymes which dissociate upon binding pCMB in an all or none fashion (*cf.* Smith and Schachman, 1971), the hexameric form of NDP-kinase bound a substantial amount of the added pCMB without significant dissociation. For instance after incubation of 15 μg of the enzyme (equivalent roughly to 0.15 nmol) with 2 nmol [^{14}C]pCMB and centrifugation in a sucrose gradient, bound radioactivity was recovered in a peak which corresponded to the undissociated hexamer and contained practically all the enzyme activity (Figure 3).

Maleylation and citraconylation yielded low molecular weight components corresponding to a monomer (mol wt 17,000) and a dimer (mol wt 34,000), as determined by sucrose gradient centrifugation. Dissociation of NDP-kinase by SDS or citraconic anhydride is a reversible process and enzymatically active hexamer was recovered on reassociation of subunits. SDS binds reversibly to all proteins (BSA for instance

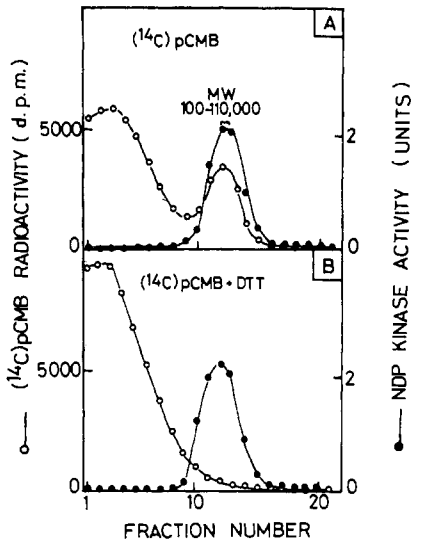

FIGURE 3: NDP-kinase (15 μg) was incubated for 1 hr at 4° with 10^{-5} M [^{14}C]pCMB in 200 μl of 100 mM triethanolamine buffer (pH 8) (expt A). An aliquot fraction of 150 μl was layered on a 5–20% sucrose gradient in 100 mM triethanolamine (pH 8), and centrifuged for 17 hr at 38,000 rpm in a SW 39 Spinco rotor at 4°; 200-μl fractions were collected for determination of radioactivity and NDP-kinase activity. Expt B was carried in the same way as expt A except that after incubation with pCMB, 5 μl of dithiothreitol (DTT) was added to a final concentration of 1 mM and let to stand for 30 min at room temperature. Centrifugation of untreated NDP-kinase (not shown on the figure) exhibited the same profile as in A and B with roughly the same total enzyme activity. In all cases, the recovery of enzyme activity from the gradient with respect to the input was 55–65%.

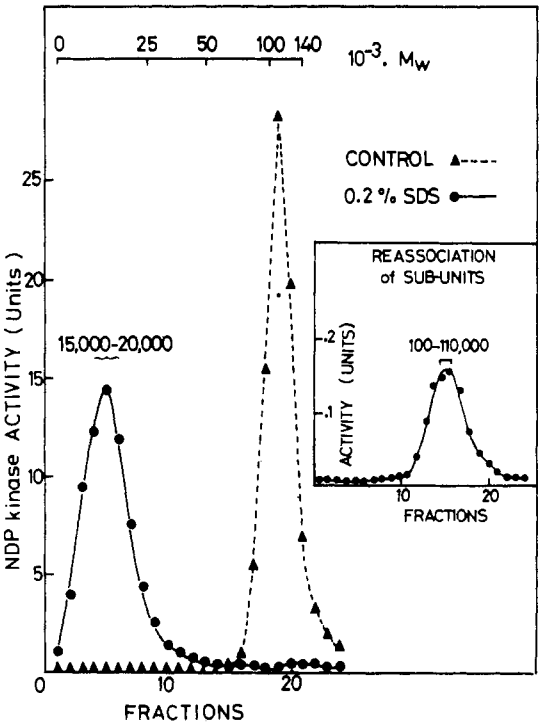

FIGURE 4: Dissociation of NDP-kinase by SDS. Reassociation of subunits after addition of BSA. NDP-kinase (125 μg) was incubated in 0.1 M triethanolamine-HCl buffer (pH 8) with 1% mercaptoethanol and 0.2% SDS in a total volume of 140 μl for 1 hr at 28°. An aliquot of 100 μl was layered on a 5–20% sucrose gradient in 50 mM phosphate buffer (pH 8), 0.2% SDS. Chymotrypsinogen (mol wt 25,000), creatine kinase (mol wt 82,000), and lactate dehydrogenase (mol wt 140,000) were used as markers in a similar gradient without SDS. The centrifugation was performed in a SW 50 rotor for 14 hr at 42,000 rpm. The temperature was maintained at 20° to avoid any precipitation of SDS; 200-μl fractions were collected and NDP-kinase activity was tested with the standard isotopic test (*cf.* Experimental Section). For the reversal 30-μl aliquots of each fraction were incubated with 100 μl of 10% BSA for 45 min at 28° before the enzyme assay. Insert: an aliquot (100 μl) of the fraction corresponding to the maximal NDP-kinase activity, after centrifugation in the presence of 0.2% SDS, was incubated with 300 μl of 10% BSA for 45 min at 28°. After a twofold dilution with distilled water, an aliquot fraction of 100 μl was layered on a 5–20% sucrose gradient and centrifuged as above except that temperature was 4°.

binds 100% of its weight of SDS, Pitt-Rivers and Impiombato (1968)) and was removed from SDS treated NDP-kinase by adding a large excess of BSA. As shown in Figure 4, subunits of 15,000–20,000 molecular weight isolated by sucrose gradient centrifugation after treatment with 0.2% SDS (SDS present in the gradient) were inactive, but exhibited a substantial phosphotransferase activity after addition of BSA to the assay medium. Evidence for reassociation of subunits to give an active hexamer on addition of BSA was obtained by rerunning a control centrifugation in a sucrose gradient; as shown in the insert of Figure 4, the NDP-kinase activity was essentially located in a region of the sucrose gradient corresponding to a molecular weight of 100,000–110,000.

Citraconylation is reversed at acidic pH (Dixon and Perham, 1968). In the case of beef heart NDP-kinase, reversal was carried out at pH 5, since at lower pH the enzyme was readily inactivated. The presence of ATP appeared to be an important factor for the recovery of enzyme activity after citraconylation. As shown in Figure 5, when citraconylation was carried out in the absence of ATP, acidification to pH 5 resulted in only a small recovery of activity. When citraconylation was carried out in the presence of 1 mM ATP, subsequent incubation at pH 5 led to a nearly total recovery of the enzyme activity. Experiments to compare the effects of ATP and ADP on citraconylation showed that ADP was 50% less efficient than ATP at a concentration of 1 mM. It is possible that ATP protects the kinase from denaturing effects of the citraconylation treatment. This protective effect of ATP may be due to either its binding to the catalytic site of the enzyme and/or to the formation of the stable phospho derivative of NDP-kinase. Sucrose gradient centrifugation assays have revealed that more dimers than monomers accumulated when citraconylation was performed in the presence of ATP. These dimers, although inactive by themselves, reassociate into an active hexamer at pH 5. The fact that "reactivatable" dimers accumulate in the presence of ATP suggests that these dimers may reflect a functional association of subunits.

ATP reacts with beef heart NDP-kinase to convert the enzyme to a stable phospho derivative (Colomb *et al.*, 1972b). In phospho-NDP-kinase, phosphate is bound to the N_1 of the imidazole ring of an histidine residue. The instability of the phospho–histidine linkage makes it difficult to measure the exact number of phosphate binding sites per mole of enzyme. However, since the breakdown of the phospho enzyme is first order (Figure 6), it has been possible to estimate the total number of [^{32}P]phosphate bonds by extrapolation (see Experimental Section). A maximal number of 3 mol of [^{32}P]-phosphate bound per mol of enzyme was found. This value, which agrees with that found for bovine liver NDP-kinase (Walinder *et al.*, 1969), for pea seed NDP-kinase (Edlund *et al.*, 1969), and for yeast NDP-kinase (Garces and Cleland, 1969), is much less than the theoretical number of six assuming that each subunit in the whole hexamer possesses one ATP binding site. Although a gross underestimation due to the technique used cannot be excluded, it is equally plausible that

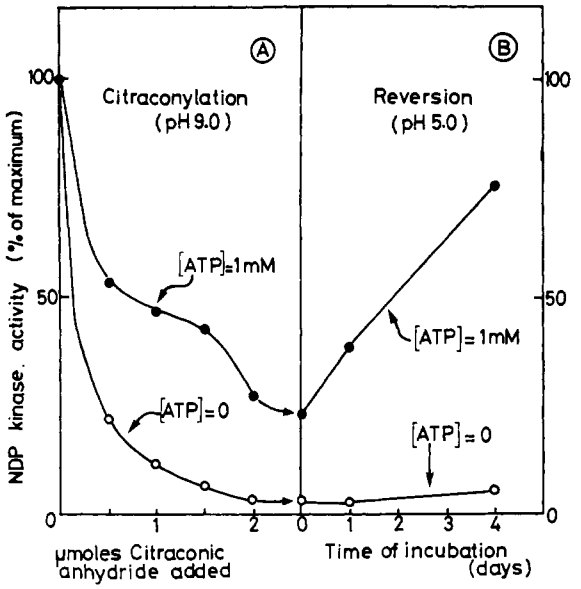

FIGURE 5: Citraconylation of NDP-kinase. Effect of ATP. Citraconylation was performed by four successive additions of 5 μl of 0.1 M citraconic anhydride in benzene to a medium containing 36 μg of NDP-kinase in 200 μl of 0.1 M phosphate buffer (pH 8.0), 40 μl of 2 M borate buffer (pH 9.0), and 10 μl of water or 10 μl of 25 mM ATP. Temperature was 28°. After each addition the anhydride was thoroughly mixed with the medium in a Vortex agitator and let to stay for 5 min before the next addition of anhydride. (A) The NDP-kinase activity was followed by the spectrophotometric assay. (B) After the fourth addition of citraconic anhydride, citraconylation was reversed by shifting the pH to 5 by addition of 50–60 μl of 1 M citrate buffer with or without 1 mM ATP.

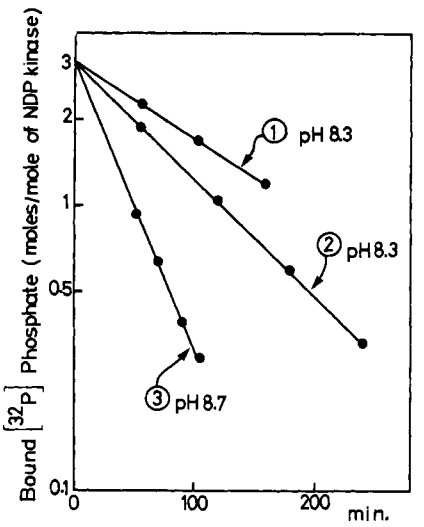

FIGURE 6: Phosphorylation of NDP-kinase by ATP; evaluation of the number of phosphates bound. Phosphorylation. In expt 1 and 3, 28 μg of NDP-kinase was incubated for 5 min at 28° with 0.02 mM [^{32}P]ATP in 150 μl of 0.025 M Tris-glycine buffer (pH 8.3) and 0.06 mM $MgCl_2$. At the end of incubation 5 μl of 0.1% Bromophenol Blue and 25 μl of 50% sucrose were added to the medium from which 30-μl aliquots were used for electrophoresis assays. In expt 2, conditions of incubation were similar to those described for expt 1 and 3 except that the concentrations of [^{32}P]ATP and $MgCl_2$ were increased by three- and fivefold, respectively. Electrophoresis and ^{32}P counting were carried out as described in the Experimental Section. The pH of the buffer (25 mM Tris-glycine) was 8.3 in expt 1 and 2 and 8.7 in expt 3.

our experimental data are accurate, and that NDP-kinase exhibits the phenomenon of "half of the sites reactive" which has been reported for some oligomeric enzymes (*cf.* Levitzki *et al.*, 1971), so that only half subunits can react with NTP to be phosphorylated.

ADP as an Inhibitor in the Transphosphorylation Reaction. In a previous paper, analysis of the kinetics of the ADP–ATP exchange reaction catalyzed by partially purified preparations of beef heart NDP-kinase (300–400 units/mg) showed that MgADP behaved not only as substrate for the phosphorylated enzyme but also as a competitive inhibitor of the reaction of ATP with the free enzyme.

Experiments presented here were carried out with a crystalline enzyme of high specific activity (1600–2500 units/mg). The K_i^{ADP} determined with this purified enzyme was much lower (five to ten times) than that previously reported, although the values of K_M^{ADP} and K_M^{ATP} were not modified. Transphosphorylation reactions with two pairs of nucleotides (ATP–ADP and UTP–ADP) were carried out with a fixed ATP or UTP to ADP ratio (α ratios). Equation 3 (Colomb

$$\frac{1}{v} = \frac{1}{V_{max}}\left[1 + \frac{K_M^{ATP}}{K_i^{ADP}}\frac{1}{\alpha} + \frac{K_M^{ADP}}{K_i^{ATP}}\alpha + (K_{ATP} + \alpha K_{ADP})\frac{1}{(ATP)}\right] \quad (3)$$

et al., 1972a) relates the rate of the ADP–ATP exchange to the concentration of ATP, taking into account the competitive inhibition of ADP with respect to ATP. A similar equation applies for the ADP–UTP exchange. Since we found inhibition by excess ADP, but not by excess ATP or UTP, the term K_M^{ADP}/K_i^{ATP} or K_M^{ADP}/K_i^{UTP} was omitted. As shown in Figure 7A (ATP–ADP exchange) plots of $1/v$ *vs.* 1/ATP at various α ratios were linear in agreement with the postulated competition between ADP and ATP for the free form of NDP-kinase. The same observation holds for the UTP–ADP exchange. The vertical intercepts and slopes obtained at various ratios have been replotted against $1/\alpha$ and α respectively (Figure 7B and C). From the new horizontal and vertical intercepts, one can derive the following constants for the ATP–ADP exchange: K_M^{ATP} = 0.5 mM, K_M^{ADP} = 0.09 mM, K_i^{ADP} = 0.02 mM (Figure 7). For the UTP–ADP exchange, they are K_M^{UTP} = 0.6 mM, K_i^{ADP} = 0.10 mM, K_i^{ADP} = 0.02 mM. The inhibition by ADP is characterized by

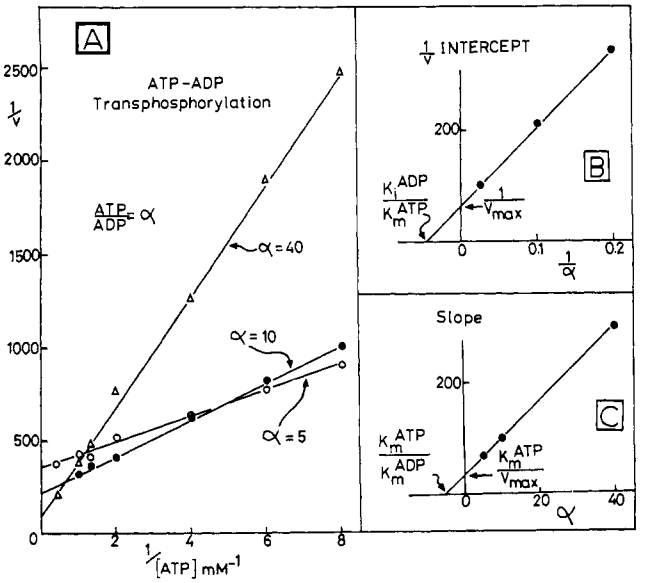

FIGURE 7: Double reciprocal plot of $1/v$ *vs.* 1/ATP for the [^{32}P]-ADP–ATP exchange. The enzyme activity was determined by the isotopic test (*cf.* Experimental Section). The rate of exchange is given in micromoles of [^{32}P]ADP exchanged per min.

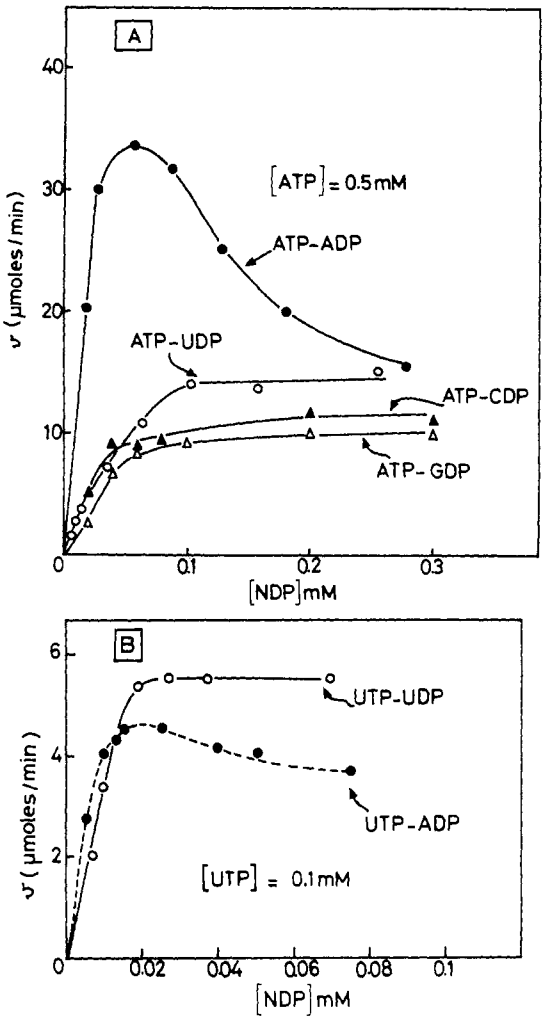

FIGURE 8: Inhibitory effect of excess ADP and NDP with [^{14}C]ATP (A) and [^{14}C]UTP (B) as phosphate donors. The enzyme activity was determined by the enzymatic test. The rate of exchange is given in micromoles of [^{14}C]ADP and [^{14}C]UDP formed per min.

a number of features. (1) It is specific for ADP. No such marked inhibition is displayed by other nucleoside diphosphates (Figure 8). (2) It is independent of the nucleoside triphosphate used; as mentioned above the K_i^{ADP} has the same value whether the nucleotide triphosphate (NTP) is ATP or UTP. (3) It is competitive with respect to NTP, pointing to the formation of a dead end complex between ADP and the free form of NDP kinase. (4) The ratio K_i^{ADP}/K_M^{NTP} which reflects the degree of competition between NTP and ADP is independent of the nature of NTP.

It is noteworthy that the K_M^{ATP} is of the same order as the K_M^{UTP}, for this implies that the difference in the rate of transphosphorylation found with different NTP species (ATP–ADP exchange is three times faster than UTP–ADP exchange) is not a result of a change in affinity of the enzyme, but is probably due to a change in its turnover.

Differential Effects of Low Concentrations of SDS and pCMB on K_i^{ADP} and K_M^{ADP} of NDP-kinase. A possible interpretation of the above data on the inhibitory effect of ADP is that beef heart NDP-kinase possesses two binding sites for ADP, one to which ADP binds as a substrate, the other to which ADP binds as an inhibitory ligand. This hypothesis is supported by the finding that K_i^{ADP} and K_M^{ADP} are differentially altered by various chemical treatments (low concentrations of SDS and of pCMB) applied to the enzyme (Table I).

TABLE I: Modification of K_M^{ADP} and K_i^{ADP} upon Addition of SDS and pCMB.[a]

Expt	Pretreatment	K_M^{ADP} (mM)	K_i^{ADP} (μM)	V_{max} (%)
1	None	0.09	9	100
	0.015% SDS	0.06	14	80
2	None	0.13	20	100
	0.015% SDS	0.08	33	75
3	None	0.10	19	100
	1% SDS			0
	1% SDS then 10% BSA	0.14	38	50
	1% SDS + 1% HSEtOH, then 10% BSA	0.09	29	55
	1% HSEtOH + 10% BSA	0.08	19	100
4	None	0.06	10	100
	10^{-7} M pCMB	0.06	18	95
	10^{-6} M pCMB	0.05	35	90
	10^{-5} M pCMB	0.05	55	89

[a] 200 μl of NDP-kinase (20 μg/ml) in 100 mM triethanolamine buffer (pH 8) was preincubated with SDS or pCMB for 10 min at 28°. A control was carried out in the same conditions. After cooling at 0° for 15 min an aliquot fraction was diluted with the triethanolamine buffer for enzyme assay by the standard isotopic test. When indicated, mercaptoethanol (HSEtOH) was added at the same time as SDS; BSA was added after preincubation with SDS ± HSEtOH and the mixture was let to stand at 28° for 45 min before the enzymatic test.

Reynolds and Tanford (1970) have reported that a wide variety of proteins bind identical amounts of SDS, on a weight to weight basis, the binding of SDS being primarily hydrophobic in nature. A pretreatment of NDP-kinase at 25° for 10 min with 0.015% SDS (ratio of SDS to protein = 7.5), which did not dissociate the enzyme as shown by sucrose gradient centrifugation, lowered the K_M^{ADP} by 30–40% and increased the K_i^{ADP} by more than 50% (Table I, expt 1 and 2). The decrease in V_{max} which accompanies the binding of SDS was pH dependent and was more marked when preincubation with SDS was performed at pH lower than 7, which suggests that not only hydrophobic, but also ionic interactions, are involved in the binding of low concentrations of SDS to NDP-kinase.

Higher concentrations of SDS (>0.1%) dissociated and inactivated NDP-kinase. As mentioned above (*cf.* Figure 4) reassociation of subunits to give an active hexamer could be achieved by addition of BSA. In expt 3, Table I, SDS was used at a concentration of 1% which totally inactivated NDP-kinase. A subsequent addition of BSA resulted in a 50% recovery of the activity with a partial loss of affinity for ADP both as a substrate and as an inhibitor. When dissociation was carried out with SDS plus mercaptoethanol, the subsequent treatment by BSA resulted in a complete recovery of the affinity for ADP as substrate (K_M^{ADP}); however the K_i^{ADP} remained 50% higher than in the control.

NDP-kinases from various sources have been found to contain SH groups capable of reacting with pCMB. As shown above, beef heart cytosol NDP-kinase in the absence of denaturant binds up to 6 mol of pCMB per mole of enzyme.

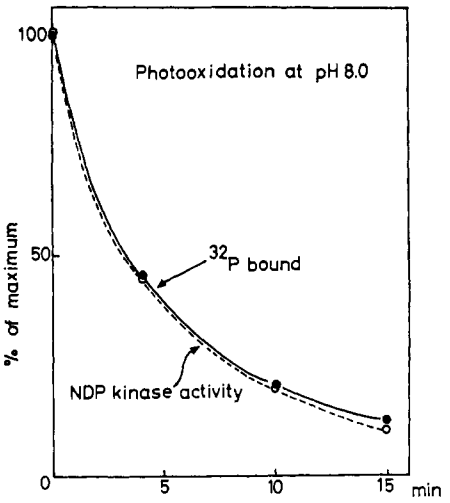

FIGURE 9: Effect of photooxidation on the activity and the phosphorylation of NDP-kinase; 5 μg of NDP-kinase in 2 ml of 0.1 M phosphate buffer (pH 8) supplemented with Rose Bengal and 0.1 mM EDTA was submitted to photooxidation at 25° as described in the Experimental Section. Aliquots of 500 μl were withdrawn at 4, 10, and 15 min for the determination of the enzyme activity (standard isotopic test) and for measurement of phosphorylation of the enzyme with [^{32}P]ATP. Phosphorylation of NDP-kinase was performed on a 450-μl aliquot to which 20 μl of 2.6 mM [^{32}P]ATP (20,000 cpm/nmol) and 10 μl of 60 mM $MgCl_2$ were added. [^{32}P]-Phospho-NDP-kinase was separated from unreacted [^{32}P]ATP on a Bio-Gel P_4 in 0.1 M phosphate buffer (pH 8) (Colomb *et al.*, 1972b).

The effect of increasing concentrations of pCMB, up to 10^{-5} M, on K_M^{ADP} and K_i^{ADP} is reported in Table I, expt 4. Below 10^{-5} M pCMB inactivation was negligible. Whereas pCMB below 10^{-5} M did not modify the K_M^{ADP}, it markedly increased the K_i^{ADP} (by 3.5-fold at 10^{-6} M pCMB and by 5.5-fold at 10^{-5} M pCMB). In all the experiments presented in Table I, the K_M^{ATP} remained virtually unchanged. In summary, it may be concluded that SDS or pCMB treatments of beef heart NDP-kinase decrease its affinity for ADP as an inhibitory ligand, but the affinity for ADP as a substrate is either not altered (pCMB) or slightly increased (0.015% SDS). The selective loss of affinity of ADP for its regulatory site is reminiscent of the phenomenon of desensitization in allosteric mechanism.

At 10^{-7}–10^{-5} M, pCMB increased by 20–30% the rate, v, of the ADP–ATP exchange, as determined by the standard isotopic assay (not corrected for ADP inhibition; see Experimental Section), although the value of V_{max} (corrected for the ADP inhibition) was slightly diminished. This increase in velocity (not corrected) is in agreement with data of Goffeau *et al.* (1968) on liver NDP-kinase and can be rationalized in terms of a decrease in the binding affinity of ADP as an inhibitory ligand, the affinities of ATP and ADP as substrates remaining unchanged.

Conformational Changes Induced by Low Concentration of SDS. It has been previously reported that photoinactivation of NDP-kinase is not pH dependent in a range of pH from 5 to 9 (Colomb *et al.*, 1972b), in spite of the fact that a histidine residue at the active site of NDP-kinase is phosphorylated by ATP and that ATP protects against photoinactivation (Colomb *et al.*, 1972b). The activity of NDP kinase and its ability to be phosphorylated by ADP fall in a parallel manner on photooxidation (Figure 9) although histidine is known to be more readily photooxidized at alkaline pH than at acid pH. The explanation put forward previously was that the histidine residue at the active site of NDP-kinase was unprotonated even at pH as low as 5 due to a lipophilic environment

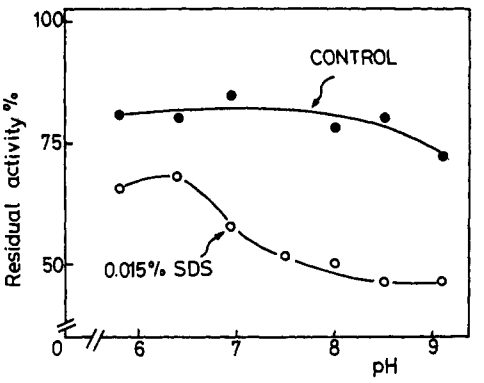

FIGURE 10: Effects of SDS on the pH dependence of NDP-kinase inactivation. NDP-kinase (20 μg/ml) was photooxidized for 3 min at 25° in the presence of 10^{-5} M Rose Bengal, 0.1 mM EDTA, and as indicated 0.015% SDS in a borate or phosphate buffer. A 50 mM borate buffer was used for pH above 8.5 and a 100 mM phosphate buffer for pH below 8.5. The media were brought to the same ionic strength with NaCl as checked by resistivity measurement. After suitable dilution, NDP-kinase activity was tested by the standard isotopic test.

(Colomb *et al.*, 1972b). As shown in Figure 10, treatment by 0.015% SDS (SDS to NDP-kinase ratio of 7, g/g) induced a pH dependence of the photoinactivation of NDP-kinase, the enzyme being more readily inactivated above pH 7 than below pH 7. It may be assumed that the histidine residue is buried in a hydrophobic environment in the active site of native subunits but becomes accessible to water on treatment with SDS, thus allowing a pH-dependent protonation of the imidazole ring.

Structural modifications of NDP-kinase upon addition of small concentrations of SDS may be related to some unexpected data on the fluorescence polarization of the tryptophan residues in NDP-kinase. As shown in Figure 11, SDS at low concentrations significantly increased both the intrinsic intensity and the degree of polarization of the fluorescence. This increase in fluorescence polarization at low SDS con-

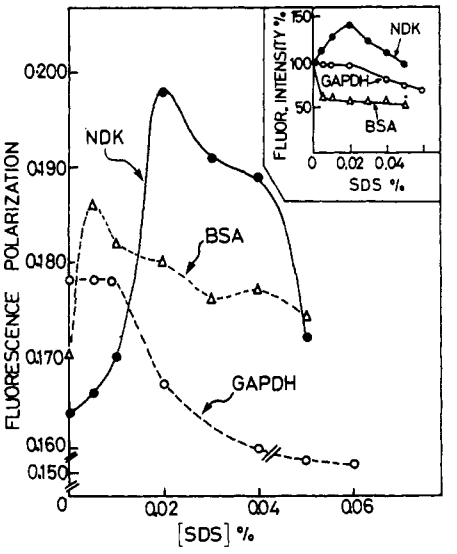

FIGURE 11: Effects of SDS on the fluorescence polarization of NDP-kinase. The following amounts of protein were used in 1.5 ml of 100 mM triethanolamine-HCl buffer (pH 8): NDP-kinase, 150 μg; GAPDH, 200 μg; BSA, 150 μg. Excitation was at 292 nm, emission at 350 nm. A correction was applied for the dilution due to SDS additions. The variation of fluorescence intensity *vs.* the concentration of SDS is reported in the insert.

centration may be due to a lower rotational mobility of the NDP-kinase molecule related to a modification of the quaternary structure of NDP-kinase, possibly an increase in size due to a partial release of the binding interactions between subunits. The other proteins examined as controls (GAPDH as an example of an oligomeric enzyme, and BSA as a monomeric protein) showed either no change or a decrease in polarization (Figure 11).

Discussion

Results presented in this paper point to the specific inhibitory effect of ADP on the transphosphorylation reaction between nucleoside tri- and diphosphates catalyzed by beef heart NDP-kinase. After extensive purification and crystallization, the enzyme exhibits a much higher affinity for ADP as an inhibitor than for ADP as a substrate; the K_i^{ADP} is some 5–10 lower than for the partially purified enzyme studied previously (Colomb *et al.*, 1972a).

Mild chemical treatments (incubation with low concentrations of SDS or pCMB) which do not significantly dissociate NDP-kinase into subunits selectively increase the value of K_i^{ADP}. This result recalls the desensitization effect in allosteric proteins and may be interpreted on the basis of two distinct ADP binding sites: a catalytic site (shared with NTPs) and a regulatory (or inhibitor) site. The catalytic site binds alternately NTP and N′DP (classical Ping-Pong mechanism). Since SDS and pCMB react with different functional groups in proteins, their similar effect on the catalytic properties of NDP-kinase is probably due to a slight nonspecific denaturation which prevents the normal interaction between the catalytic and inhibitory sites.

The decrease in ADP affinity for its inhibitor site upon addition of low concentrations of SDS is accompanied by the appearance of a pH dependence of photoinactivation typical of histidine. This is interpreted in terms of exposure to the aqueous medium of a histidine residue formerly buried in an hydrophobic environment, possibly in domains of bonding between subunits. This residue may be the same histidine which is phosphorylated by ATP, since both the overall transphosphorylation reaction and the first step which consists of the phosphorylation of NDP-kinase at the level of a histidyl residue (Colomb *et al.*, 1972b) are photoinactivated at the same rate and to the same extent.

The above model based on a classical Ping-Pong mechanism with an allosteric control is not exclusive. A two-site Ping-Pong mechanism is also possible. In the latter, one would suppose that there exists two separate catalytic sites, one for NTP, and one for N′DP. The histidyl residue which is phosphorylated by NTP (Colomb *et al.*, 1972b) might well be situated between these two sites. Competition between substrate and products might be expected to occur in each of the two partial reactions of the overall transphosphorylation catalyzed by NDP-kinase, *i.e.*, between NTP and NDP or between N′TP and N′DP (*cf.* "Introduction"). However, the difference between ADP and other NDPs in terms of their inhibitory effects cannot reside exclusively in such a simple competitive interaction, for the same K_i value for ADP is found both in ATP–ADP exchange and in UTP–ADP exchange. One possible way to rationalize these data would be to assume that ADP inhibits NTP binding, by an indirect interaction, through its binding to the N′DP site of the free enzyme.

It is not yet known whether each subunit within the whole hexamer catalyzes the transphosphorylation between NTP and N′DP or whether the functional unit in the phosphorylation reaction is the dimer. The accumulation of dimers capable of being reactivated when dissociation of NDP-kinase by citraconylation is carried out in the presence of ATP, and the presence of only three phosphorylation sites, are suggestive of the latter mechanism.

Comparison between the quaternary structure of beef heart cytosol NDP-kinase and that of other NDP-kinases deserves a few comments. In a recent paper, Palmieri *et al.* (1973) have reported that NDP-kinase from brewer's yeast is composed of six identical subunits with a molecular weight of 17,300 ± 500. A similar hexameric structure has been found for beef heart cytosol NDP-kinase. The yeast enzyme (Palmieri *et al.*, 1973) possesses a single shielded SH group per subunit. There are also SH groups essential for catalysis in NDP-kinases isolated from erythrocytes (Mourad and Parks, 1966) and from beef liver mitochondria (Pedersen, 1968). In the case of the beef liver enzyme, two classes of SH groups have been demonstrated (Pedersen, 1968); four SH groups are apparently free to react in the native enzyme, and a second set of four hidden SH groups become free to react upon denaturation. Beef heart NDP-kinase contains six SH groups readily titrated by pCMB. Incubation with 6 M urea for 1 hr at 50° results in a two- to threefold increase in the number of titrated SH groups (unpublished data), pointing to the occurrence of buried SH groups, as in the case of the liver enzyme. Palmieri *et al.* (1973) has drawn attention to the very stable hexameric structure of the yeast NDP-kinase, suggesting that this structure is stabilized by unusually large hydrophobic forces. The beef heart enzyme appears to be also endowed with a stable structure since no significant dissociation of the enzyme occurred in the presence of SDS with a weight ratio (g/g) of SDS to protein as high as 7 in our experimental conditions.

Thompson and Atkinson (1971) have shown that the reaction catalyzed by NDP-kinase responds to the energy charge of the cellular adenylate pool. They postulated that the inhibitory effect of ADP results from the binding of ADP to the substrate site of the enzyme followed by the nonproductive phosphorylation of ADP to ATP; this gratuitous reaction would represent a means by which the cell controls the exchange of phosphate between ATP and other nucleotide pools and preserves a concentration of ATP sufficient for vital reactions in metabolism (see also Colomb *et al.*, 1972a). The experimental evidence provided in the present paper indicates that the control by ADP of phosphate exchange between nucleotides is probably more complex. The consequence of the ADP inhibitory effect at the cellular level is that transfer of phosphate between ATP and nucleoside diphosphates such as UDP, CDP, and GDP occurs efficiently only under conditions where the concentration of ADP is maintained at the lowest possible level. Raising the ADP concentration in the cell results, through the inhibition of NDP-kinase, in a kind of kinetic compartmentation between adenine nucleotides and the pool of other nucleotides. This kinetic compartmentation may well be relevant to the central role of ATP in the cell economy, and its occurrence may be expected to help to maintain the intracellular concentration of ATP.

References

Butler, P. J. B., Harris, J. I., Hartley, B. S., and Leberman, R. (1969), *Biochem. J. 112*, 679–689.

Chen, R. F., Edelhoch, H., and Steiner, R. F. (1969), *in* Physical Principles and Techniques of Protein Chemistry, Part A, Leach, S. J., Ed., New York, N. Y., Academic Press, pp 208–211.

Cleland, W. W. (1963), *Biochim. Biophys. Acta 67*, 173–187.
Colomb, M. G., Chéruy, A., Duplaa, A. M., and Cordina, C. (1973), 9th International Congress of Biochemistry, Stockholm, Abstract 2m6.
Colomb, M. G., Chéruy, A., and Vignais, P. V. (1969), *Biochemistry 8*, 1926–1939.
Colomb, M. G., Chéruy, A., and Vignais, P. V. (1972a), *Biochemistry 11*, 3370–3378.
Colomb, M. G., Chéruy, A., and Vignais, P. V. (1972b), *Biochemistry 11*, 3378–3386.
Darnall, O. W., and Klotz, I. M. (1972), *Arch. Biochem. Biophys. 149*, 1–14.
Dixon, H. B. F., and Perham, R. N. (1968), *Biochem. J. 109*, 312–314.
Duée, E. D. (1968), *Bull. Soc. Chim. Biol. 50*, 1215–1219.
Edlund, B. (1971), *Acta Chem. Scand. 25*, 1370–1376.
Edlund, B., Rask, L., Olsson, P., Walinder, O., Zetterqvist, O., and Engström, L. (1969), *Eur. J. Biochem. 9*, 451–455.
Gabriel, O. (1971), *Methods Enzymol. 22*, 578–604.
Garces, E., and Cleland, W. W. (1969), *Biochemistry 8*, 633–640.
Goffeau, A., Pedersen, P. L., and Lehninger, A. L. (1968), *J. Biol. Chem. 243*, 1685–1691.
Hurlbert, R. B. (1957), *Methods Enzymol. 3*, 793–805.
Jakoby, W. B. (1971), *Methods Enzymol. 22*, 248–266.
Levitzki, A., Stallcup, W. B., and Koshland, D. E. (1971), *Biochemistry 10*, 3371–3378.
Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236*, 1372–1379.
Mourad, N., and Parks, R. E., Jr. (1966), *J. Biol. Chem. 241*, 271–278.
Palmieri, R., Yue, R. H., Jacobs, K. H., Maland, L., Wu, L., and Kuby, S. A. (1973), *J. Biol. Chem. 248*, 4486–4499.
Pedersen, P. L. (1968), *J. Biol. Chem. 243*, 4305–4311.
Pedersen, P. L. (1973), *J. Biol. Chem. 248*, 3956–3962.
Pitt-Rivers, R., and Impiombato, F. S. (1968), *Biochem. J. 109*, 825–830.
Ratliff, R. L., Weaver, R. H., Lardy, H. A., and Kuby, S. A. (1964), *J. Biol. Chem. 239*, 301–309.
Reynolds, J. A., and Tanford, C. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 1002–1007.
Sedmak, J., and Ramaley, R. (1971), *J. Biol. Chem. 246*, 5365–5372.
Shuster, L. (1971), *Methods Enzymol. 22*, 412–433.
Smith, G. D., and Schachman, H. K. (1971), *Biochemistry 10*, 4576–4588.
Thompson, F. M., and Atkinson, D. E. (1971), *Biochem. Biophys. Res. Commun. 45*, 1581–1585.
Walinder, O., Zetterqvist, O., and Engström, L. (1969), *J. Biol. Chem. 244*, 1060–1064.
Weber, K., Pringle, J. R., and Osborn, M. (1972), *Methods Enzymol. 26*, 3–42.
Westhead, E. W. (1972), *Methods Enzymol. 25*, 401–409.
Yamamoto, N., Horiuti, Y., Nishikawa, K., and Horio, T. (1972), *J. Biochem. (Tokyo) 72*, 599–613.

Studies on Spinach Leaf Ribulosebisphosphate Carboxylase. Carboxylase and Oxygenase Reaction Examined by Immunochemical Methods†

Mikio Nishimura and Takashi Akazawa*

ABSTRACT: Rabbit antisera were developed against large and small subunits (A and B) of spinach leaf ribulosebisphosphate carboxylase. Specific cross-reactivity of anti-A and anti-B sera against respective antigenic subunits was proven by: (i) quantitative immunoprecipitation analysis using Sephadex G-200 column eluates, (ii) double immunodiffusion on agar plate, and (iii) sodium dodecyl sulfate polyacrylamide gel electrophoresis of antigen–antibody complexes. Specific inhibitory effect of anti-A sera on the ribulosebisphosphate carboxylase reaction provided an additional proof for the catalytic role of the larger subunit in the enzyme catalysis. Disappearance of the Mg^{2+}-induced optimum pH shift of the enzyme reaction by the anti-B-sera-treated enzyme preparation supported our previous notion of the regulatory role of the smaller subunit. A concomitant loss of the ribulosebisphosphate oxygenase activity shown by the anti-A-sera-treated carboxylase demonstrated that the larger subunit of the enzyme molecule shared the catalytic site for both the carboxylase and the oxygenase reactions. The anti-B-sera-treated enzyme preparation showed a marked shift of optimum pH in the oxygenase reaction to a neutral side, regardless of the presence of Mg^{2+} in the assay mixture. Experimental results altogether led to a definite conclusion that the catalytic function resided in the larger subunit (A) and the regulatory function in the smaller subunit (B) of the fraction 1 protein of chloroplasts.

Ribulosebisphosphate carboxylase[1] (EC 4.1.1.39) from spinach leaf comprises two different subunits (A and B) (Rutner and Lane, 1967; Sugiyama and Akazawa, 1970). The molecular weight is 5.4×10^4 for subunit A and 1.3×10^4 for subunit B (Nishimura *et al.*, 1973). An octameric

† From the Research Institute for Biochemical Regulation, Nagoya University, School of Agriculture, Chikusa, Nagoya 464, Japan. *Received January 21, 1974.* This is paper XXIV in the series Structure and Function of Chloroplast Proteins. This investigation was supported in part by a research grant from the Toray Science Foundation (Tokyo).

[1] Abbreviations used are: RuP_2, ribulose bisphosphate; ammediol, 2-amino-2-ethyl-1,3-propanediol; SDS, sodium dodecyl sulfate; PMB, *p*-chloromercuribenzoate.